AD_____

Award Number: DAMD17-99-1-9070

TITLE: USF-1 as an inhibitor of mammary gland carcinogenesis

PRINCIPAL INVESTIGATOR: Darryl L. Hadsell, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

REPORT DATE: September 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2002	Annual (1 Sep 2001 - 31 Aug 2002)

4. TITLE AND SUBTITLE
USF-1 as an inhibitor of mammary gland carcinogenesis

5. FUNDING NUMBER
DAMD17-99-1-9070

6. AUTHOR(S)
Darryl L. Hadsell, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030
email - dhadsell@bcm.tmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
The hypothesis tested in these studies is that overexpression of USF in the mammary glands of transgenic mice will inhibit myc-dependent tumorigenesis. Expression of a FLAG-tagged form or USF-2 was targeted to the mammary gland under the control of the mouse mammary tumor virus (mmtv) long terminal repeat. Of eight lines of transgenic mice that were generated, one demonstrated expression of flag-tagged USF-2 in the lactating mammary gland at levels 12-fold over that of endogenous USF-2. Evaluation of tumorigenesis in these mice (n=17) out to 453 days of age suggests that USF-2 when overexpressed by itself is not oncogenic. In contrast, 72% of mmtv-myc mice (n=18) analyzed have developed mammary tumors with an average latency of 156±33 days. Tumor frequency and latency in bigenic mmtv-USF-2/mmtv-C-myc mice out to 200 days of age was similar to that in mmtv-C-myc mice. In contrast the growth of these myc-dependent tumors in bigenic mice was significantly lower ($P<0.01$) than that in mmtv-C-myc mice. These data support the conclusion that while overexpression of USF-2 has minimal impact on normal mammary gland development and the initiation of mammary tumors in response to C-myc overexpression, it can slow the growth of established C-myc-dependent mammary tumors.

14. SUBJECT TERMS
breast cancer, myc, transgenic mice

15. NUMBER OF PAGES
9

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Upstream stimulatory factor (USF) consists of two helix-loop-helix/zipper (bHLH/zip) proteins, USF-1 and -2, which are highly conserved among species and related to c-*myc* (1) transcription factors. Previously published cell culture studies with cancer cell models show that USF is both antiproliferative and can antagonize c-*myc* (2). The research described in this proposal addresses the idea that expression/activity of USF is a determining factor in tumor initiation and/or growth. This idea was to be explored by testing the hypothesis that targeted overexpression of USF-2 in the mammary glands of MMTV-*myc* transgenic mice will cause withdrawal from the cell cycle and differentiation thereby preventing tumors. The overall approach was to make and characterize transgenic mice that overexpress USF-2 under the control of the mouse mammary tumor virus long terminal repeat. Once in hand this new line of transgenic mice would be crossed with a previously described line of transgenic mice that overexpress c-*myc* in the mammary gland. A decrease in tumor frequency and/or an increase in tumor latency among mice that carry both MMTV-*myc* and MMTV-USF-2 as compare with those which just carry MMTV-*myc* would confirm the hypothesis.

Body

The approved statement of work for this project described two specific tasks to be completed over a 36-month period. The first task was to determine the effect of mammary-specific USF-2 overexpression on mammary gland development and lactation. This was to be completed during months 1 through 24. The second task was to determine the ability of mammary-specific overexpression of USF-2 to prevent *myc*-induced mammary tumors. This task was to be completed during months 9 through 36.

During the first 12 months of the funding period, the transgene construct was made and injected into FVB mouse embryos. This resulted in eight independent lines of transgenic mice. The major focus of task 1 for months 12 through 24 of the funding period was to complete a screen for transgene expression in lactating mammary tissue from mmtv-USF-2 lines and to determine the impact of such expression on mammary gland development and lactation. The results of this screen revealed that 2 of the 8 original lines of transgenic mice expressed the transgene only during lactation. Of these, one line expressed the transgenic protein at 8 to 12 fold over that of endogenous USF-2. This line was used for further studies on lactation and tumorigenesis.

Because the transgene was only expressed during lactation, studies on virgin development in the mmtv-USF-2 transgenic mice were not conducted. However, an analysis of the impact of the MMTV-USF-2 transgenic on lactation was conducted during months 12 through 24. This analysis revealed that overexpression of USF-2 had little impact on the development of the mammary gland, lactation, or on the abundance of milk proteins in the mammary gland. The completion of task 1 was accomplished during the months 24 to 36 with the histochemical comparison of transgene expression in the mammary tissue of nontransgenic and MMTV-USF-2 mice during lactation. Through immunofluorescent staining for USF-2 we demonstrated the dramatic elevation of USF-2 within the mammary cell nuclei of transgenic mice (Fig 1A) as compared to that of nontransgenic mice (Fig 1B).

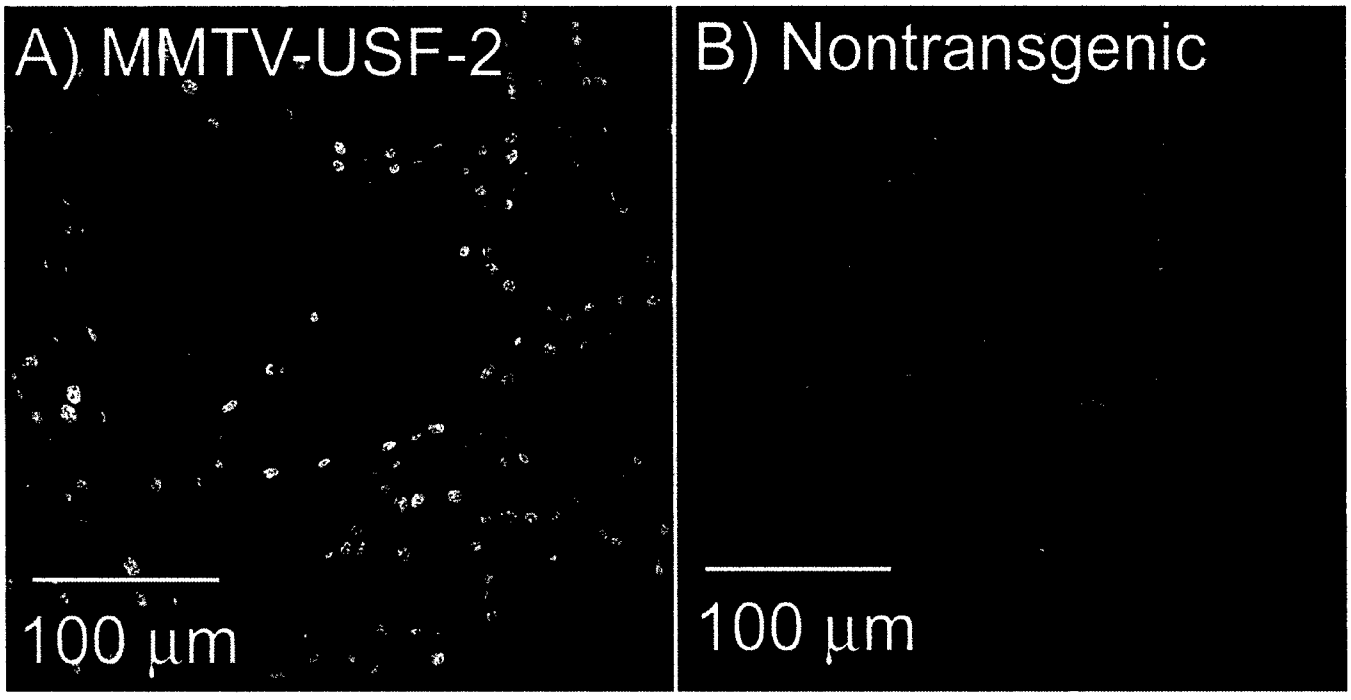


Fig 1. Immunofluorescent staining demonstrates a tremendous increase in nuclear USF-2 in mammary tissue from lactating MMTV-USF-2 mice. Frozen sections were prepared from mammary tissue collected at nine days of lactation from MMTV-USF-2 (A) or nontransgenic (B) mice. These sections were stained with antibodies against USF-2 (green/yellow), and α-smooth muscle actin (blue) and with the nuclear counterstain propidium iodide (red). Magnification is 400x.

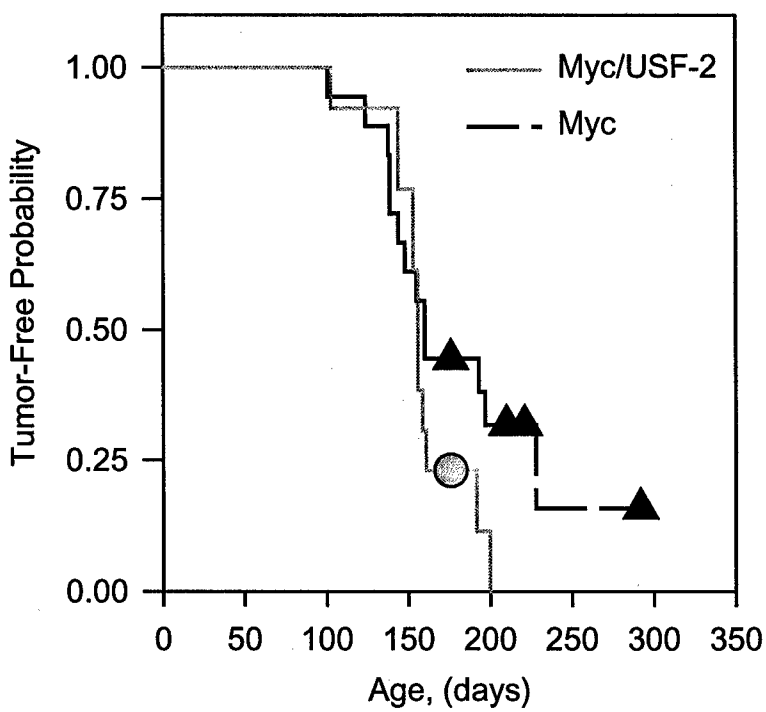
Work on a modified version of task 2 commenced during month 15 and consisted of making genetic crosses between the mmtv-USF-2 mice and two other strains of transgenic mice; mmtv-myc (4) and mmtv-v-Ha-ras (5). The goal of these studies to generate 20 female mice for each of five different genotypes (Table 1). At the termination of the sample collection period for this study, we were able to obtain a sample number that was 65 to 90 % of our original goal for all but one of the strains. In addition, the comparison of tumorigenesis among the Myc/Ras and USF-2/Myc/Ras mice was limited by the fact that surprisingly few tumors were observed in the 15 Myc/Ras females studied and even fewer trigenic USF-2/Myc/Ras females were obtained for study. Despite these limitations, the analysis of the tumor frequency data (Table 1) along with the comparison of tumor-free survival curves (Fig 2) among the Myc and Myc/USF-2 mice supports the conclusion that sample size was adequate to test our original hypothesis concerning the ability of USF-2 to inhibit myc-dependent mammary tumorigensis. This analysis demonstrated that tumor frequency, and latency were similar among Myc and Myc/USF-2 mice. Based on this analysis, we reject our initial hypothesis in favor of the alternative that overexpressed USF-2 is incapable of inhibiting myc-dependent mammary tumorigenesis.

Table 1. Summary of tumorigenesis study of transgenic mice that overexpress different combinations of USF-2, c-myc and v-Ha-ras.

genotype	*Mice obtained*	*Age[1], (days)*	*Pregnancies*	*Tumors*	*Latency, (days)*
mmtv-USF-2	17/20	453	4±3	0/17	--
mmtv-myc	18/20	292	2±2	13/18	156±33
USF-2/myc	13/20	200	2±1	12/13	156±24
myc/ras	15/20	399	<1	3/15	195±43
USF-2/myc/ras	6/20	362	1±1	2/6	230±17

[1]Age of oldest animal studied

Fig 2. Tumor—free survival is similar among Myc and Myc/USF-2 mice. Kaplan-Meier plots were constructed from tumor-free survival data collected from female MMTV-Myc (black triangles) or MMTV-Myc/MMTV-USF-2 (grey circles) mice housed continuously with a male. Censored data is illustrated by the symbols. Censored animals are those that remained tumor-free at the conclusion of the study.



In addition to collecting tumor frequency and latency data, tumor growth was measured and samples of both tumor tissue and adjacent noninvolved mammary glands were collected for further analysis. Comparison of Hematoxylin & Eosin-stained (H&E) tissue section demonstrated similar morphological characteristics among the Myc and Myc/USF-2 tumors. In general, tumor morphology was similar to the previously described cribiform morphology attributed to tumors in MMTV-Myc mice. Cells within the tumors contain two patterns of nuclei. Those with vesicular pale nuclei were often organized as solid pseudotubules with expansive

and intraductal growth. Cells with small, uniform Hematoxylin-stained nuclei were frequently found as solid sheets. All tumors had frequently observed mitotic figures along with tremendous amounts of cell shedding. In most of the tumors, massive areas of secretion/necrosis were present (Fig 3 A). In areas where this massive secretion/necrosis were not present, there were frequent small lacunae (Fig 3A) of apoptotic cells. The area occupied by necrosis within the tumors was estimated from tif images collected for each of the tumors and found to be similar among Myc and Myc/USF-2 tumors. In addition, the number of apoptotic lacunae per average field within these H&E-stained sections was similar among Myc and Myc/USF-2 tumors.

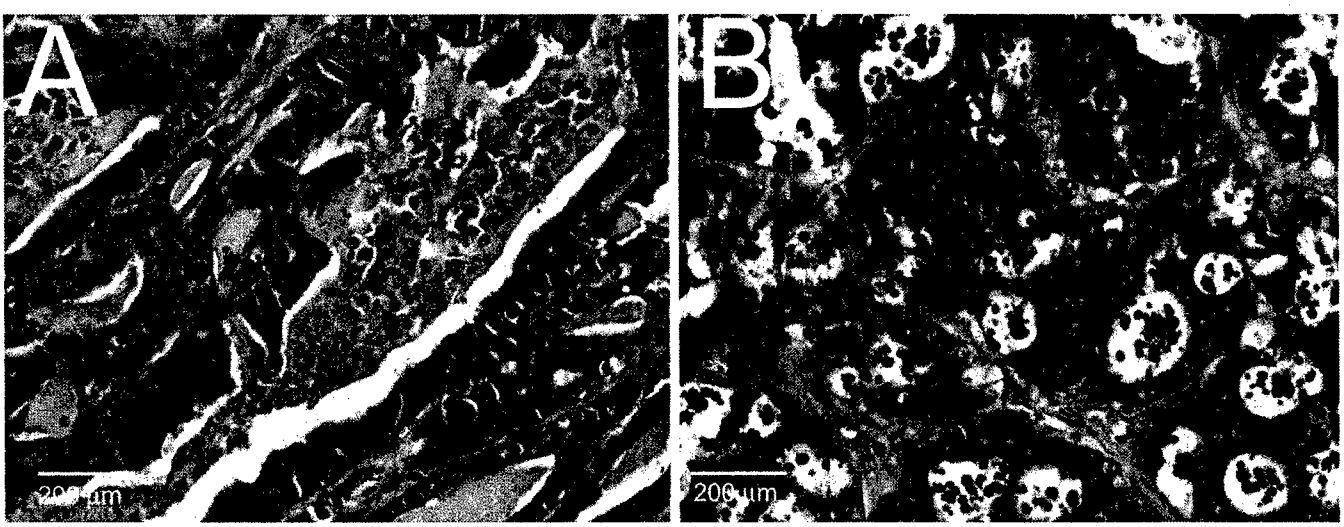


Fig 3. Morphology is similar among Myc and Myc/USF-2 tumors. Tumor samples were fixed overnight in 4% paraformaldehyde, embedded in paraffin, sectioned and then stained with H& E. the Pictures in A and B are representative of 14 myc and 14 Myc/USF-2 tumors. Magnification is 400X.

To determine if overexpressed USF-2 was capable of influencing tumor growth, weekly caliper measurements were made on the tumors for 4 weeks following their detection (Fig 4). The average tumor radius was estimated from two perpendicular measurements made on each tumor. This radius was then used to calculate tumor volume by the formula$V=4/3\pi r^3$. The results of this analysis demonstrated that the tumors in both populations grew slowly during the first two weeks and then began to grow exponentially during the last two weeks of the study period. The results also demonstrated that Myc tumors were significantly larger than Myc/USF-2 tumors by week 3 of the study. After 4 weeks of growth, the myc tumors were 49% larger ($P<0.01$) Myc/USF-2 tumors (Fig 4). This result supports the conclusion that overexpression of USF-2 slowed the growth of tumors in the MMTV-Myc mice. The results further suggest that USF-2 may have inhibited cell proliferation or increased the rate of apoptosis in the tumors of the bigenic mice.

To compare cell proliferation in the Myc and Myc/USF-2 tumors, frozen sections from each tumor were stained with an antibody specific for the G2M-phase marker, phospho-Histone H3 (). Staining for this marker was detected by immunfluorescence. Likewise apoptosis was detected by fluorescent TUNEL staining. For both analyses, the double-

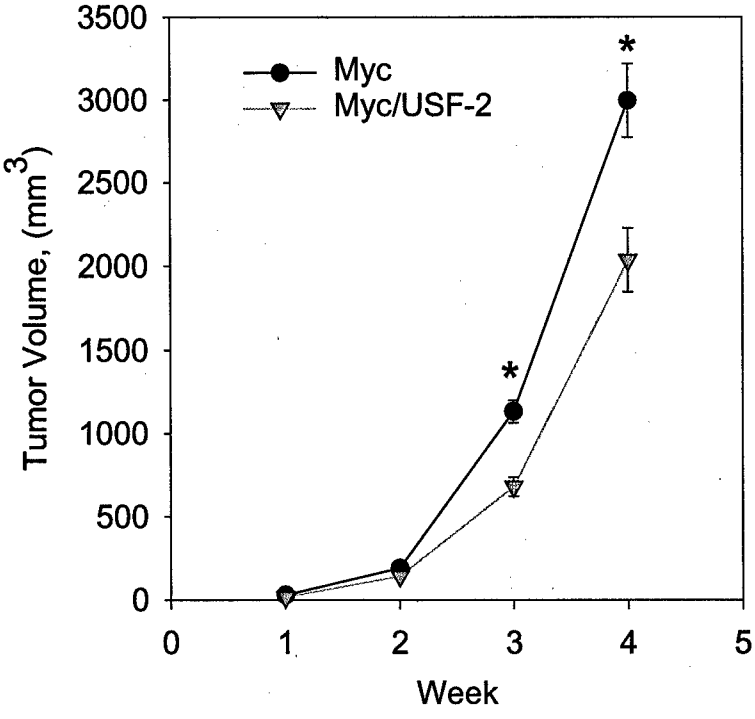


Fig 4. Overexpressed USF-2 slows the growth of Myc-dependent tumors. Tumor size was measured once per week for 4 weeks in Myc (black circles) and Myc/USF-2 (grey triangles) mice. tumor volume was estimated as described in the text. Each point represents the mean±SEM for 14 tumors. Asterisks indicate significant differences ($P<0.05$).

stranded DNA-binding dye TOPRO 3 was used as a nuclear counterstain. Estimates of the percent positive cells were obtained by automated counting of 15 TIF images collected at random from each specimen. This analysis failed to detect statistically significant differences in tumor cell proliferation or apoptosis (Fig 5). For phospho-Histone H3, the percent positive cells was very similar ($P>0.05$) among Myc and Myc/USF-2 tumors. The amount of apoptosis in Myc/USF-2 tumors was also similar to that of myc tumors..As a consequence, the diminished growth observed in Myc/USF-2 tumors is currently unaccounted for by changes in proliferation or cell death.

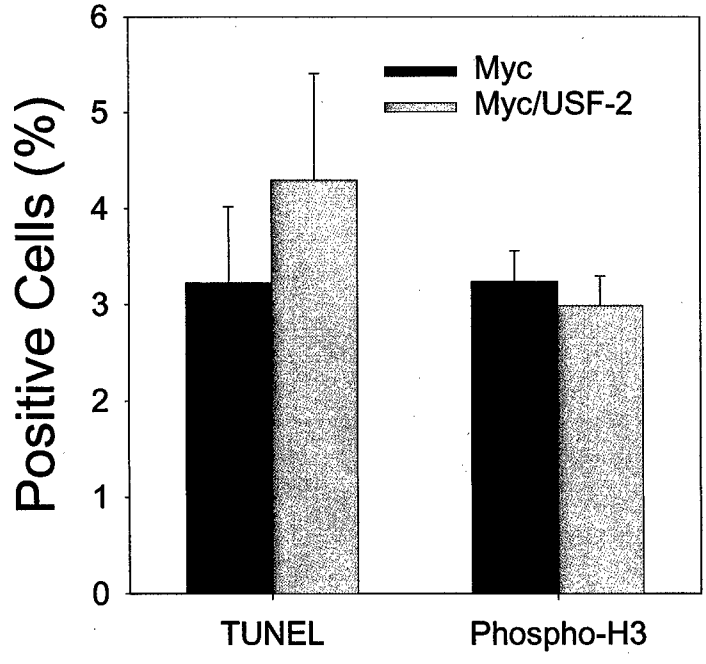


Fig 5. Overexpression of USF-2 has does not significantly influnce apoptosis or proliferation in Myc-dependent mammary tumors. Frozen sections were prepared from tumors arising in Myc (black) or Myc/USF-2 (grey) transgenic mice. Apoptosis was detected by fluorescent TUNEL. Proliferation was detected by immunofluorescent staining for phospho-histone H3 (phospho-H3). Percent positive cells was estimated by automated counting of tiff images collected from each tumor.

Key Research Accomplishments

- Determined that overexpression of USF-2 in the mammary glands of transgenic mice does not cause mammary tumors.
- Determined that overexpression of USF-2 in the mammary glands of MMTV-Myc mice has no effect on the incidence or latency of mammary tumors.
- Determined the overexpression of USF-2 in the mammary glands of MMTV-Myc mice causes a modest but statistically significant reduction in the growth of established tumors.

Reportable Outcomes

- Produced a bank of tumors and mammary tissue samples in transgenic mice that overexpress either Myc, or both Myc and USF-2.
- Presented an Abstract at the 2002 Era of Hope Meeting in Orlando, Fla.

Conclusions

The data collected to date suggest that overexpression of USF-2 in the mammary gland has little, if any effect, on the overall development or the ability to lactate. The data obtained from the tumorigenesis studies, supports the conclusion that USF-2 has no impact on the ability of overexpressed Myc to cause mammary tumors and has only modest influence over the growth of these Myc-dependent tumors. This modest ability of USF to slow the growth of mammary tumors in the myc-transgenic mice is accounted for neither by changes in cell cycle progression nor apoptosis. A no cost extension was requested for this project. During this time transgene expression within the Tumors as well as rates of proliferation and apoptosis within the adjacent, non-tumor, mammary tissue will be measured.

References

1. Luo, X. and Sawadogo, M. Functional domains of the transcription factor USF2: atypical nuclear localization signals and context-dependent transcriptional activation domains. Molecular.&.Cellular.Biology., *16*: 1367-1375, 1996.

2. Luo, X. and Sawadogo, M. Antiproliferative properties of the USF family of helix-loop-helix transcription factors. Proc.Natl.Acad.Sci.U.S.A., *93*: 1308-1313, 1996.

3. Guy, C.T., Cardiff, R.D., and Muller, W.J. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. Molecular and Cellular Biology, *12*: 954-961, 1992.

4. Stewart, T.A., Pattengale, P.K., and Leder, P. Spontaneous mammary adenocarcinomas in transgenic MTV/myc fusion genes. Cell, *38*: 627-637, 1984.

5. Sinn, E., Muller, W., Pattengale, P., Tepler, I., Wallace, R., and Leder, P. Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. Cell, *49*: 465-475, 1987.

Appendices

None